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Accumulation, transformation and breakdown of DSP toxins from the toxic dinoflagellate *Dinophysis acuta* in blue mussels, *Mytilus edulis*

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Running title: DSP toxins in *Mytilus edulis*

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Abstract

Okadaic acid (OA), dinophysistoxins (DTX) and pectenotoxins (PTX) produced by the dinoflagellates *Dinophysis* spp. can accumulate in shellfish and cause diarrhetic shellfish poisoning upon human consumption. Shellfish toxicity is a result of algal abundance and toxicity as well as accumulation and depuration kinetics in mussels. We mass-cultured *D. acuta* containing OA, DTX-1b and PTX-2 and fed it to the blue mussel, *Mytilus edulis* under controlled laboratory conditions for a week to study toxin accumulation and transformation. Contents of OA and DTX-1b in mussels increased linearly with incubation time, and the net toxin accumulation was 66% and 71% for OA and DTX-1b, respectively. Large proportions (≈50 %) of both these toxins were transformed to fatty acid esters. Most PTX-2 was transformed to PTX-2 seco-acid and net accumulation was initially high, but decreased progressively throughout the experiment, likely due to esterification and loss of detectability. We also quantified depuration during the subsequent four days and found half-life times of 5-6 days for OA and DTX-1b. Measurements of dissolved toxins revealed that depuration was achieved through excreting rather than metabolizing toxins. This is the first study to construct a full mass balance of DSP toxins during both accumulation and depuration, and we demonstrate rapid toxin accumulation in mussels at realistic *in situ* levels of *Dinophysis*. Applying the observed accumulation and depuration kinetics, we model mussel toxicity, and demonstrate that a concentration of only 75 *Dinophysis* cells l^{-1} is enough to make 60 mm long mussels exceed the regulatory threshold for OA equivalents.

Keywords: Diarrhetic shellfish poisoning, Okadaic acid, Dinophysistoxin, Pectenotoxin, *Dinophysis*, *Mytilus edulis*

1. Introduction

Contamination of shellfish with various biotoxins can lead to several different shellfish poisoning syndromes following human consumption (Landsberg 2002). In all cases, the causative toxins are *de novo* produced by certain photo- or mixotrophic microalgae – not by the shellfish (Landsberg 2002, Lewitus et al. 2012). Filter-feeding transfers the toxins to the shellfish, where they may accumulate to high concentrations. Symptoms range from nausea over paralysis and amnesia to death depending on the involved toxins. Consequently, commercial shellfish harvesting (fisheries or aquaculture) are subject to extensive monitoring of *in situ* concentrations of causative algae and/or toxicity of harvested shellfish.

Diarrhetic shellfish poisoning (DSP) syndrome is one such syndrome, and the causative organisms are a frequent cause for concern in shellfish industries, as they may cause prolonged closures of mussel harvesting, sometimes lasting several months, with severe economic repercussions (Hinder et al. 2011). Predicting DSP toxicity in mussels would be a powerful mitigation tool, but has so far proved difficult (Reguera et al. 2014). Identification of the causative toxins and variability in algal toxicity has received the bulk of the attention, but shellfish toxicity is also a result of toxin accumulation and depuration kinetics. Nevertheless, comparably little is known about accumulation and depuration of DSP toxins in any shellfish species.

DSP toxins are produced by species of the two marine dinoflagellate genera *Dinophysis* and *Prorocentrum* (Hoppenrath and Elbrächter 2011; Reguera et al. 2014). The toxin producing *Prorocentrum* species are benthic, and thus unavailable for suspension-feeding mussels in most cases. Hence, *Dinophysis* is considered the main source of DSP toxins in marine shellfish. Acute effects of DSP toxins include diarrhea, nausea, vomiting and cramps, but chronic effects have also been reported, including carcinogenic effects and effects on the immune- and nervous systems and alterations in DNA and cellular components (Valdiglesias et al. 2013).

The DSP toxin group includes okadaic acid (OA) and its analogues the dinophysistoxins (DTX) as well as pectenotoxins (PTX). From the OA-group, OA, DTX-1, DTX-1b, and DTX-2 and their diol-ester precursors have been found in the toxin-producer *Dinophysis* spp. (Miles et al. 2006a, Hackett et al. 2009, Fux et al. 2011, Nielsen et al. 2013, Reguera et al. 2014). From the PTX-group, PTX-2 and PTX-11 - PTX-14 have been found in plankton samples or *Dinophysis* spp. cultures (Draisci et al. 1996, Miles et al. 2004b, Miles et al. 2006b, Suzuki et al. 2006). In shellfish, on the other hand, OA/DTX toxins are often transformed to fatty acid esters (collectively known as DTX-3), and these frequently comprise more than half the total OA/DTX in mussels (Vale and de M. Sampayo 2002a, Vale 2006). Likewise, mussels are known to transform PTX-2 to PTX-2 seco acid (PTX-2sa), but data on PTX in shellfish are scarce (Vale and de M. Sampayo 2002b). PTX-2sa may also be transformed to numerous different fatty acid esters, further adding to the complexity (Wilkins et al. 2006, Torgersen et al. 2008).

Our knowledge on accumulation of DSP toxins in mussels comes almost exclusively from field populations; controlled laboratory experiments are very scarce (Bauder et al. 1996, Rossignoli et al. 2011b). This owes primarily to the fact that culturing of *Dinophysis* spp. was only recently made possible (Park et al. 2006). Depuration (or detoxification) has been studied more intensely using mussels contaminated *in situ*. Effects of various parameters, including food availability (Blanco et al. 1999, Svensson 2003, Svensson and Förlin 2004, Marcaillou et al. 2010), temperature (Shumway and Cembella 1993, Blanco et al. 1999) and mussel size and lipid content (Svensson and Förlin 2004, Duinker et al. 2007) have been evaluated using different mussel species. Reported toxin half-life times vary substantially, from less than a day to 25 days, but despite the efforts, depuration kinetics of DSP toxins is far from well understood. Accumulation kinetics remain virtually unstudied.

With the recent discovery of suitable culturing techniques, it is now possible to study accumulation kinetics of DSP toxins from the prime *in situ* source, *Dinophysis* spp. Thus, for the first time, we studied the intoxication of mussels with OA, DTX-1b and PTX-2 supplied via mass cultured *Dinophysis acuta*. We chose the blue mussels, *Mytilus edulis*, as the target organism due to its commercial importance and well-studied physiology. The aim was to establish a mass balance under controlled laboratory conditions, quantifying accumulation, transformation, depuration and excretion of DSP-toxins from *D. acuta*. Based on these results, we model the toxin content of various-sized *M. edulis* under different *Dinophysis* spp. concentrations.

2. Materials and Methods

2.1. Culture conditions and specimen collection

A laboratory culture of the marine DSP producing dinoflagellate *Dinophysis acuta* (Strain DANA-2010) was established from water samples collected in the North Atlantic during a research cruise in June 2010 (60°24’ N; 6°58’ W) (Nielsen et al. 2013). Cells were isolated under a dissection scope by micro manipulation. The culture was non-clonal, as ≈10 cells were picked and grown together in 1 ml 0.2 µm filtered seawater. To facilitate the mixotrophic growth of *D. acuta,* the ciliate *Mesodinium rubrum* was added as prey twice a week at a prey:predator ratio of 10:1. The *M. rubrum* culture was fed the cryptophyte *Teleaulax amphioxeia* at similar intervals and ratios. All protist cultures were grown in a temperature controlled room at 15°C in f/2 seawater-based growth medium (Guillard and Ryther 1962) with a pH of 8.0 ± 0.05, a salinity (psu) of 30.0 ± 1.0 and a dissolved inorganic carbon content of 2.3 ± 0.1 mmol l^{-1}. A Sentron ArgusX pH-meter equipped with a Hot-line cupFET probe was used to determine pH (NBS scale), and salinity was measured with a handheld visual refractometer. Dissolved inorganic carbon was measured with an infrared gas analyzer as described in detail elsewhere (Nielsen et al. 2007). An irradiance of 130 µmol photons m^{-2} s^{-1} was provided by Osram 58W/640 cool-white fluorescent tubes at a 16:8 h light:dark cycle. Previous studies of the *Dinophysis acuta* culture had shown that it produced OA and PTX-2 as well as a novel isomer of DTX-1 tentatively termed DTX-1b (Nielsen et al. 2013).

The mussel *Mytilus edulis* was collected in Oresund, Denmark with a dredge deployed from boat during autumn 2011. The mussels were brought back to the laboratory, where they were kept for at least a month in a continuous flow of aerated sea water (30 ± 1.0 psu, 10°C) before use in experiments. The collection site typically has low abundances (<1 cell ml^{-1}) of *Dinophysis* spp. almost year round, but only rarely experiences blooms. The mussels were thus not naïve. On the other hand, one month in the laboratory may have removed any adaptations towards DSP toxins before the onset of the experiment.

2.2. Accumulation, transformation and breakdown of DSP toxins

The experiment was designed to study accumulation and depuration rates of different DSP toxins in *Mytilus edulis*. Several initial attempts to intoxicate *M. edilus* with DSP toxins from *Dinophysis acuta* resulted in production of pseudo-faeces and correspondingly low (<1%) net toxin retention. These observations were most likely due to high concentrations of *D. acuta* (>20,000 l^{-1}), and the present experiment was thus designed to work with low *D. acuta* concentrations.

Four replicate 12 l aquaria were setup in a 15°C temperature controlled room. Five litre GF/C filtered seawater (salinity = 30 ± 1) and 12 *M. edulis* (length 23.4 ± 1.0 mm) were added to each. Mussels were allowed 6 h to attach to the bottom, after which centrifugal aquarium pumps were deployed to ensure adequate mixing. The aquaria were all aerated with atmospheric air (≈40 ml min^{-1}). To limit the growth of *D. acuta* in both supply cultures and the four aquaria, the experiments were conducted at relatively low irradiance levels (≈10 μmol photons m^{-2} s^{-1}).

2.3. *Dinophysis acuta* inflow

Once the experiment started, *D. acuta* was pumped into each aquarium by a Gilson Minipuls 3 peristaltic pump (Biolab A/S, Denmark), equipped with a 4 channel pump head. A well-mixed *D. acuta* supply culture was ensured by continuous stirring with a magnetic stirrer. Glass funnels, custom-made from Pasteur pipettes, were positioned in the narrow intake of the tubes to avoid clogging of *D. acuta* cells. In order to design the experiment with low cell concentrations of *D. acuta*, a simple model was developed to predict the steady-state concentration of *D. acuta* as a function of *D. acuta* inflow rate:

$$A_t = \frac{A_{t-1} \times V_{t-1} + F - (M \times C \times A_{t-1})}{V_t} \qquad (1)$$

Where A is *D. acuta* concentration (cells l^{-1}) at times t and t-1, V is total water volume (l), F is inflow of *D. acuta* in (cells h^{-1}), M is number of mussels and C is clearance rate (l ind^{-1} h^{-1}). For designing the experiment, C was, according to Kiørboe and Møhlenberg (1981), assumed to be 1.0 l ind^{-1} h^{-1} for the size of *M. edulis* used.

The experiment was designed to produce an asymptotic rise to a maximum steady-state *D. acuta* cell concentration as a result of the continuous *D. acuta* inflow and the constant clearance by mussel. The inflow was set to be 1000 *D. acuta* cells $mussel^{-1}$ h^{-1}, which, according to eq. 1, would result in a steady-state *D. acuta* concentration of 1000 cells l^{-1}. The continuous food supply was maintained for a week, only interrupted by the quick daily samplings. After the first week, the food supply was stopped, and the mussels remaining in the aquaria were left without additional food. During the first week, where *D. acuta* was supplied, the removal of mussels at each sampling was accompanied by a reduction of the inflow rate of *D. acuta* according to eq. 1, and a steady inflow per mussel was thus maintained.

2.4. Sampling

One mussel was harvested from each aquarium on day 0, 1, 2, 3, 4, 7, 7½, 8, 9, 10 and 11. Shell length and wet weight of soft parts were recorded. The soft parts were transferred to cryotubes and immediately frozen at -18°C for later toxin extraction and analysis. Mussel filtering activity was inspected visually twice on each day of sampling by scoring each mussel as either open or closed.

Cell concentrations of *D. acuta* in supply cultures were determined before and after each sampling, by fixing 3 ml subsamples with Lugol's (final conc. 1%) and enumerating *D. acuta* by normal light microscopy in 1 ml Sedgwick-Rafter sedimentation chambers. Combined with volumes of *D. acuta* supply cultures before and after sampling, this allowed total inflow of *D. acuta* to be calculated.

The toxin contents of *D. acuta* were determined on day 0 and 7 by taking triplicate 0.5 ml subsamples from the supply culture. The subsamples were added to Eppendorf tubes with 0.45 µm spin filter inserts (VWR, Denmark). These were centrifuged at 800 × g for 1 minute, after which the filtrate was discarded, and the tubes with filter inserts were stored at -18°C until later toxin extraction and analysis. Water temperature and oxygen saturation were monitored daily in all four aquaria with a WTW OXI 96 oximeter equipped with an EO 96 sensor.

At each sampling, the *D. acuta* inflow was stopped, and all the water in each aquarium was replaced with 5 l of pre-filtered seawater at similar salinity, temperature and pH. For determination of cell concentrations of *D. acuta*, 100 ml subsamples of the discarded water were fixed with glutaraldehyde (final concentration 2%), and filtered onto 25 mm black polycarbonate filters (pore size 2 µm). These were mounted in immersion oil on microscopy slides, and inspected under epifluorescence microscopy (Olympus BX50) for enumeration of *D. acuta* at 100x magnification. Due to the complete exchange of water at samplings, the *D. acuta* concentrations in the aquaria changed over time; immediately after samplings, concentrations would have been close to zero, and as *D. acuta* were added, the concentrations would have increased asymptotically towards a steady-state maximum, where inflow and clearance cancelled each other out.

Dissolved toxins were sampled during the depuration period (Day 7-11) by mixing the remaining ≈5 l of discarded water with 2 g of Diaion® HP-20 resin (Sigma-Aldrich, MO, USA) in 10L glass flasks and bubbling heavily for 12 h. The resin was mixed freely with the water and collected with a 20 µm mesh following the 12 h period.

After termination of the experiment, average clearance rates could be calculated from the observed steady-state concentrations of *D. acuta* by simple reorganization of equation 1:

$$C = \frac{A_{t-1} \times V_{t-1} + F - (A \times V)}{M \times A_{t-1}} \qquad (2)$$

2.5. Control experiment without mussels

On day 7, after feeding was stopped, a control experiment was setup, to determine the reduction (or growth) in *D. acuta* without mussels. A flow of *D. acuta* similar to the highest one used in the experiment was directed into four additional aquaria with 5 l seawater, identical aquarium pumps and aeration. The control experiment lasted for 24 h, corresponding to the sampling interval in the experiment. After 24 h, cell concentrations of *D. acuta* were determined as described above, and these were compared to the total inflow of *D. acuta* during the same period.

2.6. Clearance rate with *Rhodomonas salina* as food source

A second control experiment was set up with the cryptophyte *Rhodomonas salina* as food source, to compare with the clearance rates of *M. edulis* obtained with *D. acuta* as a food source. This experiment also served to prove the principal of the clearance rate calculations. Biovolume equivalents comparable to the steady-state levels observed in the *D. acuta* experiment were set as the target (≈2000 *R. salina* cells ml^{-1}). Triplicate 10 l, aerated aquaria were added 5 l filtered seawater and 10 *M. edulis* individuals each. Mussels were allowed a few hours to attach by byssus before submersible aquarium pumps were started in order to ensure mixing. A continuous flow of 35 ml h^{-1} of a 455.000 cells ml^{-1} *R. salina* culture was provided, and the concentrations of *R. salina* in the aquaria were monitored every hour. The experiment was terminated when a steady-state concentration of *R. salina* had been attained (4 h). *Rhodomonas salina* was enumerated using a Multisizer 3 Coulter Counter (Beckman Coulter) equipped with a 100 µm aperture and with background samples subtracted. Shell length and wet weight of soft parts of each mussel was determined after the experiment was completed.

2.7. Toxin extraction and analysis.

Upon toxin extraction, 0.5 ml of circonia beads and 1.3 ml methanol were added to each cryotube-sample containing the soft parts of a mussel. These were homogenized with a FastPrep instrument (Theromo-Savant, Illkirch, france) for 45 sec at max speed (6.5 m s^{-1}). Samples were left to extract for 5 min after which they were centrifuged and the supernatant transferred to a 20 ml glass scintillation vial. An additional 1.3 ml methanol was added, and the sample was re-homogonized, -extracted and -centrifuged. The supernatant was transferred and combined with the first. A third extraction was performed in the same way, resulting in a total of ca. 3.8 ml methanol. Additional methanol was added to a total of 4.0 ml (by weight, ρ = 0.79 g cm^{-3}). Half this volume was removed for hydrolysis of toxin esters; the other half was processed without hydrolysis.

Hydrolysis of toxin esters was achieved by adding 250 µl NaOH (2.5 M) to the 2 ml methanol toxin extract and heating the samples to 76°C for 40 min. After cooling, samples were neutralized with 250 µl HCl (2.5 M).

2.7.1. Cleanup with solid phase extraction (SPE)

Milli-Q water was added to all samples before clean-up with 3 ml LC-18 SPE cartridges (Sigma-Aldrich, Germany). Four ml were added to non-hydrolyzed aliquots and 3.5 ml to hydrolyzed ones, thus bringing the total methanol content down to <33%. Organic solvent content should not exceed 50% in order for SPE cartridges to bind DSP toxins. SPE cartridges were pre-treated with 1 ml methanol for 10 min, and washed twice with Milli-Q water, before the sample extract was added, all the time taking care not to dry the cartridges out. Slowly (≈1 ml min^{-1}) the extract was passed through the SPE cartridge, and the toxin thus adsorbed onto the SPE column. The cartridges were continuously refilled with extract until all 6 ml had been added, and were then washed thrice with 1 ml Milli-Q water. Finally, the toxins were eluted with 1 ml 80% methanol directly into 2 ml glass HPLC vials.

2.7.2. Algal samples

The spin filters containing *D. acuta* were extracted with 100 µl 100% methanol for one hour after which the Eppendorf tubes and filters were centrifuged at 800 × g for two minutes. The filtrate was then transferred to a 250 µl glass insert in a 2 ml HPLC vial that was closed with a lid.

2.7.3. Dissolved toxins

Aliquots of one to two g of wet HP20 resin were weighed into 15 ml centrifugation tubes and 10 ml methanol were added to the resin and left over night. The next day the resin methanol slurry was transferred into a chromatography glass column (24 cm length, 1 cm ID) with frit topped with a 1 cm quartz sand layer and the centrifugation tube was rinsed with an additional 5 ml methanol. The column filling was topped with another 1 cm quartz sand layer and the supernatant methanol was eluted to a glass beaker. Additional 15 ml methanol was added to the column and subsequently slowly eluted (ca. 10 drops min^{-1}). Combined eluates were homogenized by vortexing and a 1 ml aliquot was used for LC-MS/MS analysis.

2.7.4. Hydrolysis of HP20 extracts and HP20 resin

10 ml of HP20 extracts from the samples taken at day 9 were mixed with 1.25 ml of 2.5 N sodium hydroxide solution and heated to 76 °C for one hour and subsequently left to cool down to room temperature overnight. The reaction mixture was neutralized with 1.25 ml of 2.5 N acetic acid and solvents removed in a rotary evaporator. The residue was adjusted with methanol to 1 ml and passed through a spin filter (0.45 µm) prior to LC-MS/MS analysis. As quantitative control an HP20 extract was treated identically, but without alkaline hydrolysis.

Approximately 5g of resin of the four same samples from day 9 were accurately weighed and suspended in 1 ml of methanol. The mixtures were treated with sodium hydroxide and subsequently neutralized as described above. After neutralization, additional 4 ml of methanol were added to the resin suspension. The supernatant was removed from the resin after centrifugation and the remaining resin was re-extracted with another 4 ml methanol. The extracts were combined and prepared for measurement as described above.

LC-MS/MS analysis followed the protocol of Nielsen et al. (2013), with the added detection of PTX-2sa at the mass transition *m/z* 894>213.

3. Results

Salinity, temperature and oxygen conditions of the four aquaria were stable and non-detrimental to the mussels (*Mytilus edulis*) throughout the experiment (table 1). Two of the initial 48 mussels died during the experiment, and this caused the experiment to be shortened from 12 to 11 days (Fig. 1A). Visual inspections confirmed that mussels were generally open (and thus active) during the first week where *Dinophysis acuta* were supplied (89 ± 5 % [mean ± S.D.] mussels scored as open). The 24 h control experiment without mussels, recorded a 21% loss of *D. acuta* cells during a 24 h period (data not shown), and this was accounted for in subsequent calculations of mussel ingestion and clearance. The *D. acuta* culture contained OA, DTX-1b and PTX-2 at cell quotas of 3.0 ± 0.5, 7.6 ± 1.3 and 39.0 ± 4.6 pg cell^{-1}, respectively, with no statistically significant differences during the experimental period.

Table 1. Experimental conditions in the four replicate aquaria and lengths and weight of the 44 *Mytilus edulis* sampled. Values are means ± SD.

Parameter	Value
Salinity (psu)	30 ± 1.0
Temperature (°C)	16.0 ± 1.8
O_2 (% sat.)	100 ± 3.5
Mussel length (mm)	23.4 ± 1.0
Mussel wet weight (g)	0.23 ± 0.05

3.1. Clearance and ingestion

The average steady-state *D. acuta* concentration reached was 6,762 ± 2,317 cells l^{-1} (Fig. 1B). Based on equation 2, this translates to a clearance rate of 0.15 ± 0.04 l ind^{-1} h^{-1} (table 2). Combined, these values demonstrate a maximum ingestion rate of 1,028 ± 240 *D. acuta* cells ind^{-1} h^{-1} once steady-state concentrations of *D. acuta* were reached.

The inflow of *D. acuta* was 17,711 ± 267 cells $mussel^{-1}$ day^{-1}, but only 10,719 ± 149 of these were cleared by the mussels (Fig. 1C). Correspondingly, on day 7, a total of 122,000 *D. acuta* cells had been provided per mussel, but only 74,000 ± 5,580 of these had been cleared. Thus, the average ingestion rate across the whole experiment was 452 ± 34 *D. acuta* cells ind^{-1} h^{-1} (compared with the maximum ingestion rates of 1,028 ± 240 *D. acuta* cells ind^{-1} h^{-1} at steady state concentrations).

Table 2. Feeding and filtration characteristics of *Mytilus edulis* in the intoxication experiment with *Dinophysis acuta* and in the control experiment using *Rhodomonas salina*. The steady-state concentration of *D. acuta* was also expressed as *R. salina* equivalent using a biovolume conversion factor of 278 *R. salina* for each *D. acuta*. Note the distinction between the steady-state ingestion rate (achieved once *D. acuta* concentrations had stabilized) and average ingestion rate over the whole experiment. Values are means ± SD.

	Steady state Cell concentration (cells ml^{-1})	*R. salina* equivalents (cells ml^{-1})	Clearance rate (l ind^{-1} h^{-1})	Steady-state ingestion (cells ind^{-1} h^{-1})	Average ingestion (cells ind^{-1} h^{-1})
D. acuta	6.8 ± 2.3	1894 ± 649	0.15 ± 0.04	1028 ± 240	452 ± 34
R. salina	1898 ± 495	1898 ± 495	0.86 ± 0.19	-	-

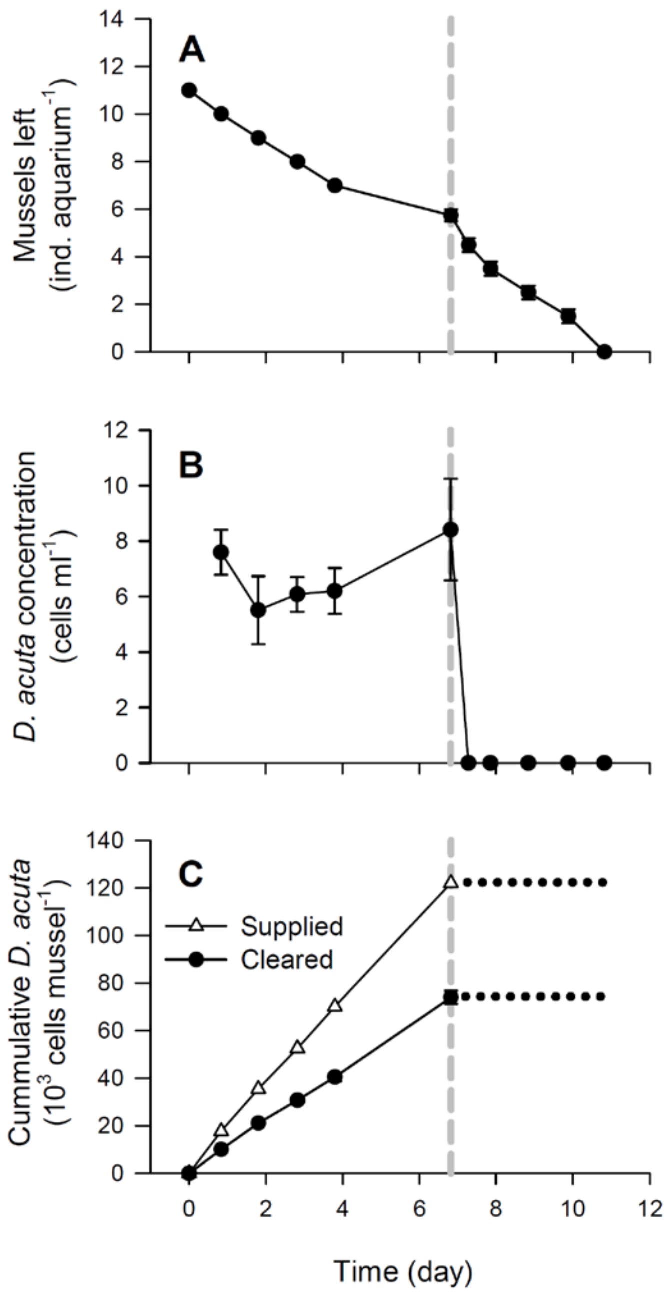

Fig. 1. Intoxication experiment with *Mytilus edulis* fed *Dinophysis acuta*. Mussel sampling scheme (A), steady-state *D. acuta* concentrations at each sampling (B) and cumulative *D. acuta* supplied and cleared (C) in the intoxication and depuration experiment with *M. edulis*. The vertical dashed line indicates when the *D. acuta* supply was stopped. Note that only the steady-state concentrations are depicted in B, not the increase between samplings. Symbols and bars are means ± SD (n=4) except for the supplied amount of *D. acuta* (C), which are values from the *D. acuta* common supply culture (n=1).

3.2. Intoxication

All three toxins clearly accumulated in *M. edulis* during the seven day intoxication period (Fig. 2). Toxin clearance and accumulation in *M. edulis* were fitted with linear regression. Based on these fits, mussels cleared 135.5 ± 1.9, 343.2 ± 4.8 and 1,760 ± 24.5 ng toxin g^{-1} mussel d^{-1} of OA, DTX-1b and PTX-2, respectively (Fig. 2 & table 3). Contents of both OA and DTX-1b (total, i.e. free+esters) increased linearly throughout the seven days of feeding, at rates of 89.79 ± 4.9 and 242.3 ± 12.0 ng toxin g^{-1} mussel d^{-1}, respectively. This corresponds to a net retention of 66% for OA and 71% for DTX-1b. Thus, approximately two thirds of the cleared OA and DTX-1b was accumulated in the mussels. Most of the PTX-2 was immediately converted to PTX-2sa in the mussels (Fig. 3 C&D), and in contrast to OA and DTX-1b, the increase in [PTX-2 + PTX2sa] content was not linear but rather resembled an asymptotic rise towards a maximum value (Fig. 2 C). Thus, the net retention of PTX-2 observed during the first day was almost 100%, but this value decreased as the experiment progressed. On the final day of feeding, day 7, it had dropped to 25% (table 2).

The majority of the accumulated OA found in *Mytilus edulis* was found as esters; only 27.1 ± 4.2 % was found as free OA (Fig. 3A). Free DTX-1b accounted for 45.9 ± 5.3 % of the total DTX-1b pool (Fig. 3B). Thus, esters of DTX-1b were less frequent compared to OA, but still comprised the majority of the total DTX-1b pool. PTX-2 and PTX-2sa were apparently both lower in hydrolyzed samples compared to non-hydrolyzed ones. This demonstrates that PTX-2 and PTX-2sa are unstable under the strong alkaline conditions present during the hydrolysis step. There may thus have been a significant pool of unquantified fatty acid esters of PTX-2 and PTX-2sa.

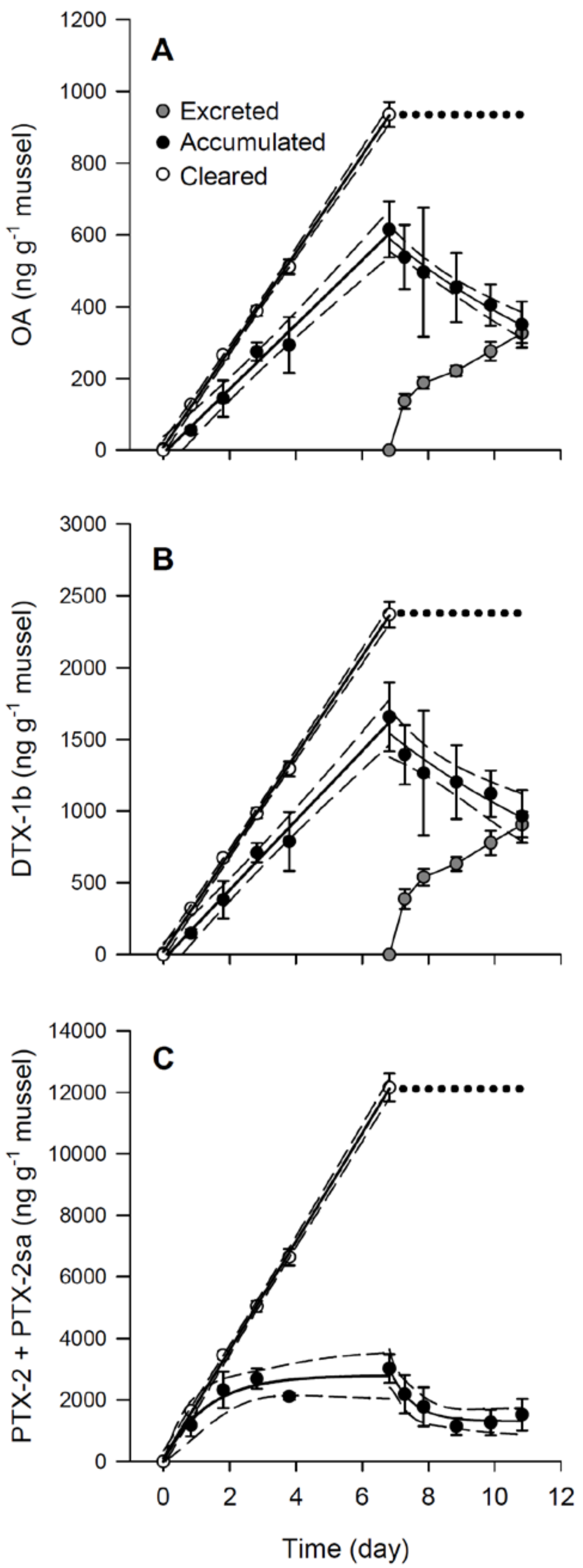

Fig. 2. Total toxin concentrations (hydrolyzed samples) of *Mytilus edulis* in the intoxication and depuration experiment compared to the amount cleared from *D. acuta*. OA (A), DTX-1b (B) and PTX-2 (C). Toxin accumulation until day 7 was fitted to a linear increase, and the subsequent toxin depuration was fitted either to a simple exponential decay function (OA and DTX-1b) or a two-compartment exponential decay model (PTX-2). Blue lines denote 95% confidence interval around the means. Also depicted are values of cumulative excreted OA (A) and DTX-1b (B) during the depuration period from day 7 onwards. This was not included for PTX-2 due to the inability to measure total PTX-2 (see text and Fig. 3). Symbols and bars are means ± SE (n=4).

Table 3. Regression parameters of DSP toxin clearance, accumulation and loss in *Mytilus edulis*. Slopes and coefficients are reported as means ± SD. TAE = toxin accumulation efficiency. $t_{½}$ = half-life of each toxin calculated as exponential decay functions with the exponential coefficients, bshown in the table. OA and DTX-1b includes fatty acid esters, and PTX-2 includes PTX-2sa, the primary form of PTX-2 in the mussels. * = TAE of PTX-2 decreased over time and the value reported here is that observed for the entire 7-day feeding period.

	Cleared		**Accumulated**			**Exponential loss**		
	r^2	Slope ($ng\ g^{-1}\ d^{-1}$)	r^2	Slope ($ng\ g^{-1}\ d^{-1}$)	TAE %	r^2	b ± S.D.	$t_{½}$ (days)
OA	0.99	136 ± 2	0.99	90 ± 5	66%	0.97	0.13 ± 0.01	5.3
DTX-1b	0.99	343 ± 5	0.99	242 ± 12	71%	0.90	0.12 ± 0.02	5.8
PTX-2	0.99	1760 ± 25	-	-	25%*	0.69	0.24 ± 0.01	2.9

3.3. Depuration

Total OA and DTX-1b contents in *M. edulis* began to decrease immediately after feeding was terminated (Fig. 2B & C). The decreases were fitted to simple exponential decay functions ($y = a \times e^{-bx}$) and these gave good r^2 values (table 3). The fitted exponential decay function yielded exponential coefficients of 0.131 ± 0.012 for OA and 0.120 ± 0.021 for DTX-1b. This corresponds to half-life times of 5.3 and 5.8 days, respectively (table 3). Depuration of [PTX-2 + PTX-2sa] was poorly described ($r^2 = 0.69$) by the simple one-compartment exponential decay function used for OA and DTX-1b. This likely owes to the difficulties measuring PTX-2sa fatty acid esters discussed later.

3.4. Dissolved toxins

Hydrolysis of the HP20 resin and HP20 resin extracts showed that toxins dissolved in the water consisted exclusively of the free-forms. No toxin esters were found in the water during the depuration period. Dissolved toxins were found during the entire depuration period (day 7-11). The cumulative amounts of dissolved (excreted) toxins increased to 326 ± 53 ng OA g^{-1} mussel and 905 ± 180 ng DTX-1b g^{-1} mussel. This slightly overestimated the observed decrease in mussel toxicity, accounting in total for 123 % and 130 % of the loss in mussel toxicity of OA and DTX-1b, respectively (Fig. 2A & B). PTX-2 and PTX-2sa were not measured in the dissolved fraction.

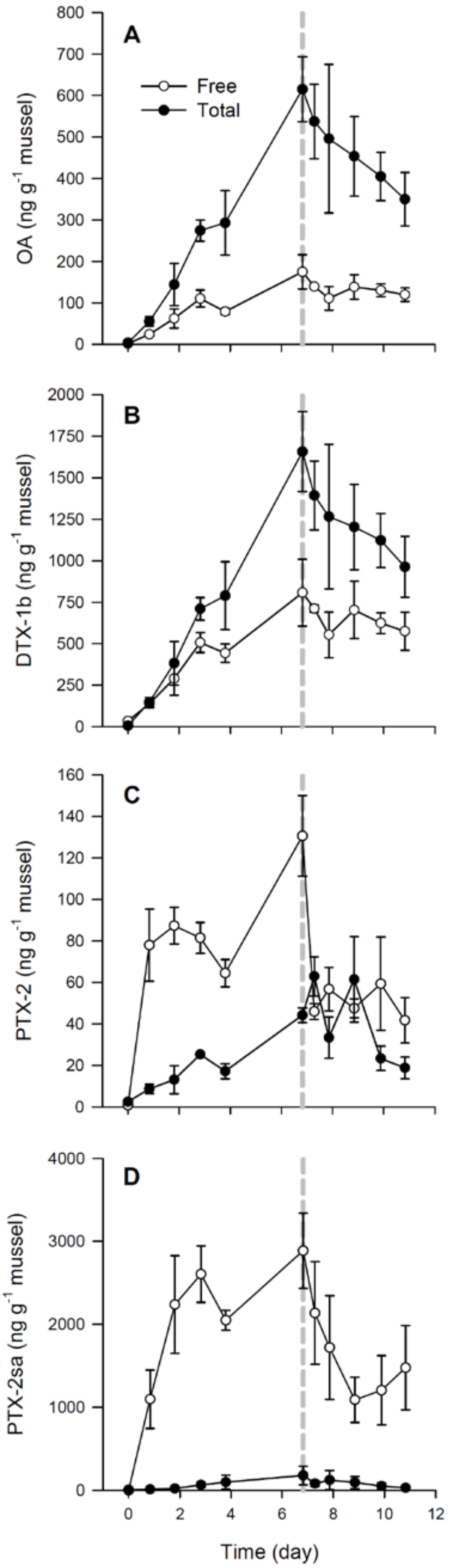

Fig. 3. Concentration of total and free toxins in *Mytilus edulis* during the intoxication and depuration experiment. OA (A), DTX-1b (B), PTX-2 (C) and PTX-2sa (D). The difference between total and free toxin represent toxin esters. Pectenotoxins are destroyed during the hydrolysis process, thus giving lower values in the 'total' samples compared to the 'free' samples. Symbols and bars are means ± SE (n=4).

3.5. Clearance rate with non-toxic prey

The *Rhodomonas salina* concentrations stabilized within the first hour of the control experiment, indicating that the mussels were active (Fig. 4). The steady-state concentrations of *R. salina* in the last three hours of the control experiment was 1898 ± 452 cells ml^{-1} (mean ± SD). From equation 2, this corresponds to an average clearance rate of 0.86 ± 0.19 l ind^{-1} h^{-1}. This was statistically significantly higher than the clearance rate of 0.15 ± 0.04 l ind^{-1} h^{-1} observed when eating *D. acuta* (t-test, $p<0.001$, n=4). The average shell length of the mussels used in the control experiment was 22.83 ± 1.58 mm and the average wet weight was 0.175 ± 0.038 g. Thus, the mussels used in the control experiment were slightly smaller than those used in the DST intoxication experiment (compare with table 1).

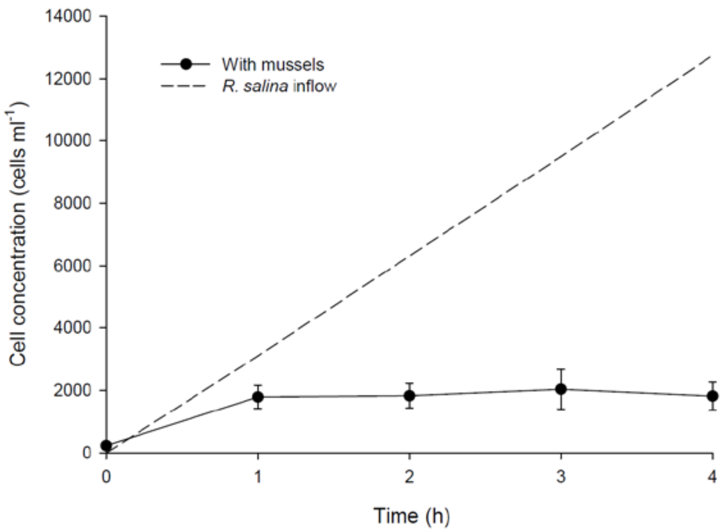

Fig. 4. Clearance of the non-toxic cryptophyte *Rhodomonas salina* by *Mytilus edulis*. The dashed line indicates cumulative *R. salina* inflow in the absence of *M. edulis*. Values are means ± SD (n=4).

4. Discussion

4.1. Accumulation

Okadaic acid and DTX are powerful inhibitors of serine/threonine protein phosphatases and may in addition cause various chronic effects (Valdiglesias et al. 2013). Pectenotoxins disrupts actin in the cytoskeleton, and may cause cell cycle arrest and cell death (Spector et al. 1999; Ares et al. 2005; Anonymous 2009). Thus, accumulation of both toxin groups is undesirable at best. Nevertheless, blue mussels, *Mytilus edulis*, used in this experiment accumulated large amounts of both OA and DTX-1b when feeding on *Dinophysis acuta*.

Mussel toxicity relative to the total amount of ingested toxins demonstrated net retention of 66 and 71 % for OA and DTX-1b, respectively. This is similar to the net retention of 63 % previously found for an unspecified mussel species, when using OA encapsulated in gelatin-acacia microcapsules (Rossignoli et al. 2011a). It is, however, much higher than the 1-10% found in the only previous study to use live dinoflagellates as prey (Bauder et al. 2001), but this study did not include toxin esters and may consequently have missed a substantial part of the toxin pool. Available evidence thus suggests that *M. edulis* accumulate the majority of both OA and DTX ingested.

PTX-2 also accumulated rapidly in the mussels (mostly as PTX-2sa), although the net retention appeared to decrease during the seven-day intoxication period, from nearly 100% after the first day to ≈25% at the end of the seven-day intoxication period. This is the first study to examine accumulation kinetics of PTX in a shellfish species. All three toxins can be transformed to various analogue compounds following ingestion by mussels, and a transformation of PTX-2 and PTX-2sa to compounds not included in the LC/MS-MS analysis could provide a compelling explanation of the apparent decrease in net retention of PTX-2 during the seven days of *D. acuta* exposure.

4.2. Transformation

OA and DTX may be transformed to various fatty acid esters analogues upon ingestion by mussels. In our experiment, approximately half the OA and DTX-1b in *M. edulis* were found as esters, and previous studies have shown high variability in the proportion of esters in various shellfish species (Vale and de M. Sampayo 2002a, Moroño et al. 2003, Vale 2006, Torgersen et al. 2008). Rossignoli et al. (2011) argued that esterification is a step in the depuration process based on the observation that almost all the OA found in faeces of *Mytilus galloprovincialis* were esters. In our experiment, however, all excreted OA and DTX-1b were on the free form – not esters. The apparent discrepancy may illustrate species specific differences, but it may also reflect differences between excretion as faeces and in the dissolved form (e.g. urine).

PTX-2sa constituted the majority of the total PTX-2 pool in mussels at all times, indicating that PTX-2 was rapidly transformed into PTX-2sa. Large fractions of PTX-2sa have been found in mussels before, and shellfish (including mussels) have previously been demonstrated to facilitate the conversion of PTX-2 into PTX-2sa (Suzuki et al. 2001a, b, Vale and de M. Sampayo 2002a, Vale 2004). Our results support this, and further demonstrate that the process is rapid, and that large amounts of PTX-2 can be transformed via this process daily, even in the small mussels examined here. Transformation to seco acid may be a protective measure as PTX-2sa has a low toxicity compared to PTX-2 (Miles et al. 2004a, 2006c)

The net accumulation of [PTX-2 + PTX-2sa] apparently decreased as the experiment progressed and by the end of the feeding period, only 25% of the ingested PTX-2 could be accounted for in the mussels. This is most likely explained by a further transformation of PTX-2sa into fatty acid esters as recently observed by others (Wilkins et al. 2006, Torgersen et al. 2008). The build-up of PTX-2sa suggests that the conversion from PTX-2sa to the different PTX-2sa esters is a slower process than the conversion from PTX-2 to PTX-2sa.

We also found that PTX-2 and PTX-2sa are unstable under the strong basic conditions present during the hydrolysis step. This is in accordance with earlier attempts to hydrolyse PTX-2 (Vale and de M. Sampayo 2002; Miles 2007; Anonymous 2009). To quantify PTX-2 esters, one can either (1) hydrolyse samples enzymatically as demonstrated by Doucet et al. (2007) or (2) include all known PTX esters in the LC-MS analysis as done by Torgersen et al. (2008). Only esters with known mass transitions can be included, however, and standards of PTX esters are not yet commercially available. Thus, with the LC/MS-MS method applied here, we most likely overlooked the majority of the total PTX-2 pool, and this should be considered when monitoring shellfish toxicity using LC/MS-MS.

4.3. Depuration

Mussel toxin contents started decreasing immediately after feeding with *D. acuta* was terminated. The calculated half-life times of 5.3 and 5.8 days for OA and DTX-1b respectively, are somewhat fast compared to literature values: Marcaillou et al. (2010) found a half-life time of 20 days for 4-7 cm long, unfed *Mytilus edulis*. However, this discrepancy could potentially be related to the size difference of the mussels used. Mussel size has previously been speculated (but not proven) to be negatively correlated with toxin depuration rate (Duinker et al. 2007). The depuration rates in the present study cover only the first few days of depuration, and if there is both a labile and a more slowly depurated toxin pool as suggested by Blanco et al. (1999) and Moroño et al. (2003), then our one-compartment model could overestimate the depuration rates.

PTX depuration is difficult to evaluate due to the fact that we did not measure PTX esters. The half-life times acquired from the applied two-compartment model are likely flawed.

Gross rates of toxin accumulation can be estimated by combining net rates of toxin accumulation with depuration rates. By the end of the feeding period, the gross accumulation accounted for 95 % and 99 % of the ingested OA and DTX-1b, respectively. Thus, effectively all ingested OA and

DTX-1b seems to be taken up in the digestive tract of *M. edulis*. No significant amount was excreted directly with remains of the food source without first being absorbed by the intestine. On the same note, the amount of excreted toxins found in the water during the depuration period accounted for all of the observed loss in mussel toxicity. Thus, we have demonstrated that *M. edulis* depurated solely through excretion of toxins rather than metabolizing. This is the first study to establish a mass balance of DSP toxins in a mussel species during accumulation and subsequent depuration. It should be mentioned, that the situation described here is a controlled laboratory experiment, where several factors diverge from *in situ* conditions. Most importantly these include, but are not limited to, initial starvation and the single-species diet offered.

4.4. Total toxicity

Okadaic acid, dinophysistoxins and pectenotoxins are currently still considered together for regulatory purposes (European Commission 2011). Using a toxicity equivalent factor (TEF) for each compound, the total toxicity is expressed in OA equivalents. The TEF of PTX-2sa is set to zero rendering this compound unregulated and excluded from a total toxicity measure. OA and PTX-2 are both regulated, whereas DTX-1b is not because it was only recently discovered and a TEF has yet to been assigned (Nielsen et al. 2013). If we assume a TEF similar to DTX-1 (TEF=1), it would be necessary to regulate DTX-1b in order to protect consumer health. Fig. 5 shows the cumulative amount of OA, DTX-1b and PTX-2 in *M. edulis* during the experiment. We included DTX-1b since it will likely add to total toxicity, but refrain from calculating a total toxicity in OA equivalents since DTX-1b does not yet have a TEF. Even under the low *Dinophysis* spp. concentrations used in the present study, mussels exceeded the regulatory limit of 160 µg kg^{-1} within the first day of exposure to *D. acuta* (Fig. 5). This was unexpected, and largely owes to the finding that practically all ingested toxin was absorbed in the intestine, leaving nothing to immediate excretion with faeces.

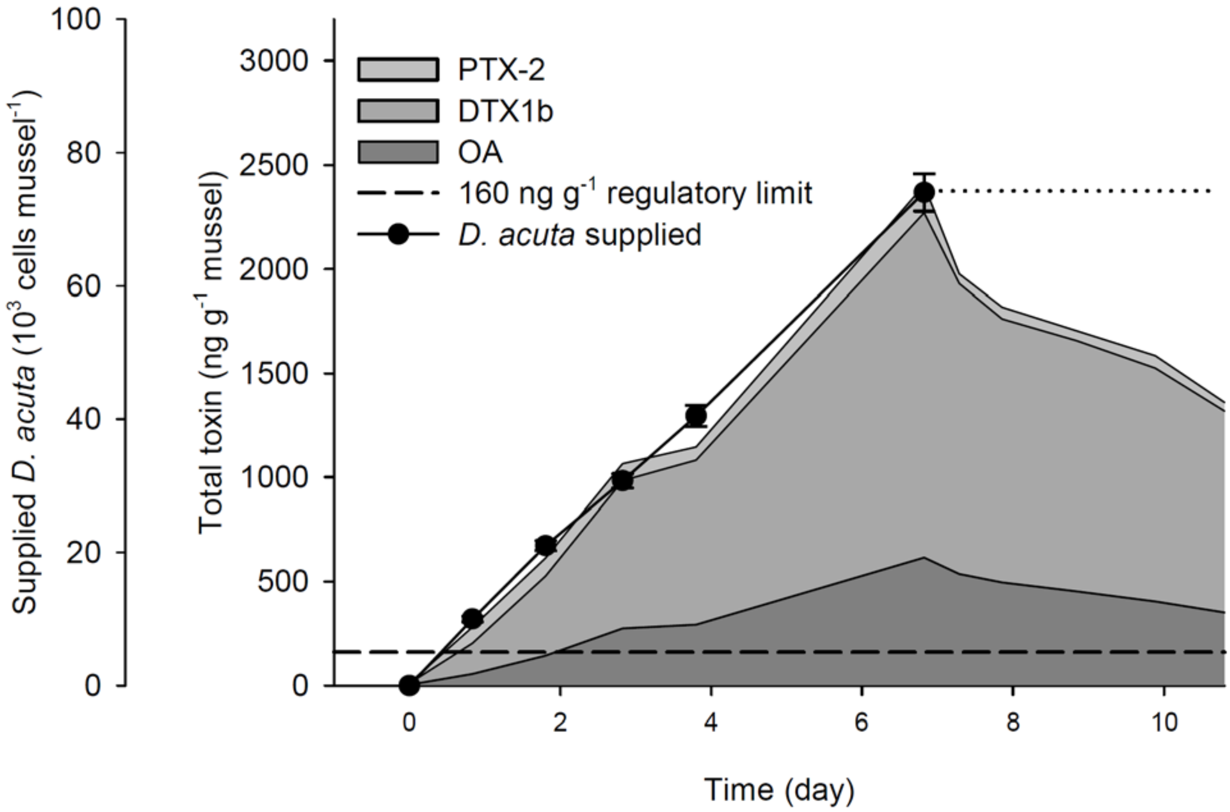

Fig. 5. Total toxin (OA + DTX-1b + PTX-2) content of *Mytilus edulis* and the cumulative amount of ingested *Dinophysis acuta* during the intoxication and depuration experiment. Mussels exceeded the combined OA+DTX+PTX regulatory limit of 160 ng g^{-1} wet weight within the first day.

4.5. Modelled toxin content

Accumulation and depuration kinetics are useful in understanding DST intoxication events of *M. edulis*. It is currently unknown, to what extent the observed toxin accumulation efficiencies and depuration rates can be extrapolated to different sized mussels in natural populations and to situations where *Dinophysis* spp. are ingested at different concentrations and in combination with other food particles. Adopting the observed accumulation and depuration kinetics (of OA and DTX-1b), and using literature values of clearance (Kiørboe and Møhlenberg 1981) and wet weight (Jones et al. 1992) of different sized mussels, one can model the toxin content of various sized mussels under different conditions (Fig. 6) Such calculations reveal, that a moderately toxic (similar to the OA+DTX-1b content of *D. acuta* in this study = 10 pg DST cell^{-1}) population at sub-bloom densities (500 *D. acuta* cells l^{-1}) would make *M. edulis* of any size exceed the regulatory threshold of 160 ng g^{-1} within only one or two days of exposure (Fig. 6A). Also, a concentration of only ≈75 *D. acuta* cells l^{-1} of moderate toxicity (10 pg DST cell^{-1}) is enough to make 60 mm long *M. edulis* exceed the regulatory threshold within ≈10 days (Fig. 6B). Due to the increased weight-specific clearance rate, smaller mussels would require even fewer *D. acuta* per litre in order to potentially exceed the threshold. Finally, as an example, a 50 mm long mussel would exceed the regulatory threshold within a day at concentrations from 500 *D. acuta* cells l^{-1}, but it may take up to 20 days to approach steady-state toxin concentrations in the mussels (Fig. 6C).

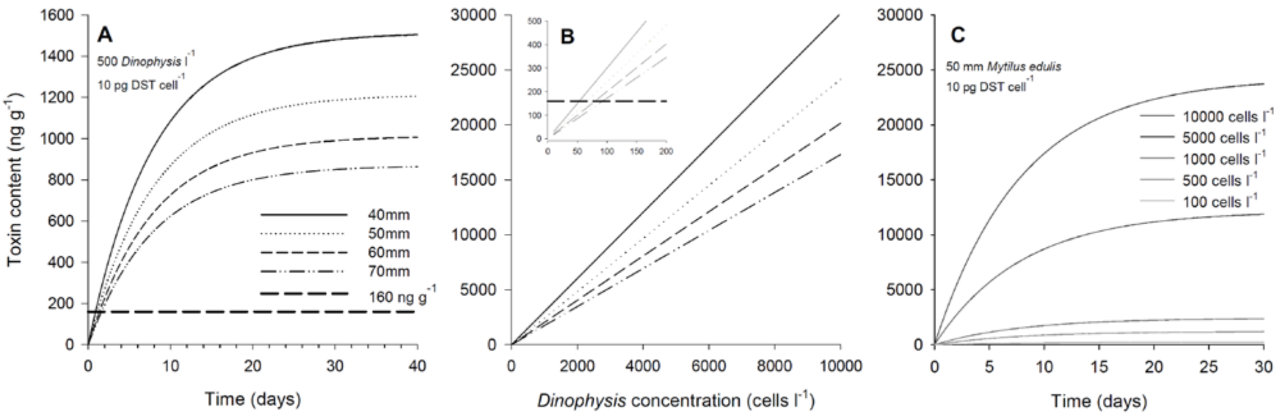

Fig. 6. Modelled toxin contents of *Mytilus edulis* based on the observed toxin accumulation efficiencies of OA and DTX-1b and literature values of clearance rates and wet weight of different sized mussels. (A) Toxin content as a function of time when subjected to a stable 500 cells l^{-1} *Dinophysis acuta* concentration of moderate toxicity (10 pg DST cell^{-1}), corresponding to the total OA and DTX-1b found for *D. acuta* in this study. (B) Maximum achievable toxin content as a function of *D. acuta* cell concentration of the same toxicity. Legend as in A. (C) Toxin content of a 50 mm long *M. edulis* at five different *D. acuta* concentrations from 100-10,000 cells l^{-1} as a function of time. Note the different y-axis scales.

4.6. Feeding physiology and effects of DSTs on mussels

Clearance rates of *M. edulis* were much lower when feeding on *D. acuta* compared to the non-toxic cryptophyte *Rhodomonas salina* (Fig. 4, table 3). Thus, intake of *D. acuta* seems to affect the filtration of *M. edulis*. Similar effects are caused by other toxic algae, including saxitoxin containing *Alexandrium* spp. (e.g. May et al. 2010), azaspiracid containing *Azadinium spinosum* (Jauffrais et al. 2012) and karlotoxin containing *Karlodinium veneficum* (Brownlee et al. 2008). Further studies are needed, however, in order to determine the extent and severity of the effect of *D. acuta* on mussel filtration, and to determine whether the effect owes to one or more of the DSP toxins.

Conflict of interest

The authors declare no conflict of interest.

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